# Detection of DNA Polymorphisms in *Homalodisca coagulata* (Homoptera: Cicadellidae) by Polymerase Chain Reaction-Based DNA Fingerprinting Methods

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**ABSTRACT** DNA polymorphisms were detected in *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae) with the following DNA fingerprinting methods: inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) and primer pair-ISSR-PCR (pp-ISSR-PCR), randomly amplified microsatellite polymophisms (RAMP), selective amplification of microsatellite polymorphic loci (SAMPL), and primer pair-random amplification of polymorphic DNA-polymerase chain reaction (pp-RAPD-PCR). But first, a small-scale DNA fingerprinting screening procedure was initiated with these methods with a few individual insects to estimate the most sensitive and efficient method(s). In total, 205 polymorphic markers were generated with the four methods. The efficiency ratio estimated the following order for each method: 1) pp-ISSR-PCR and ISSR-PCR, 2) RAMP, 3) pp-RAPD-PCR, and 4) SAMPL. The screening efficiency ratio estimated that pp-ISSR-PCR and ISSR-PCR were the most efficient methods. DNA polymorphisms were detected in a natural population of 10–30 insects. The number of polymorphic loci ranged from five (pp-RAPD-PCR reaction 6) to 32 (ISSR-PCR primer 13), and the percentage of polymorphic loci was 100% for most primers tested. DNA fingerprinting methods tested were able to detect geographic variation in populations of *H. coagulata* from Bakersfield and Riverside, CA, and Weslaco, TX. Dendrograms based on Nei's genetic distance showed that *H. coagulata* from Bakersfield and Riverside formed a cluster separate from Weslaco in three DNA fingerprinting reactions tested incorporating simple sequence repeats. DNA fingerprinting methods tested were also able to distinguish between three *Homalodisca* sharpshooters: *H. coagulata*, *Homalodisca insolita* (Walker), and *Homalodisca liturata* (Ball). The present results confirmed the utility of the DNA fingerprinting screening procedure and demonstrated, for the first time, genetic variation in natural populations of glassy-winged sharpshooters by PCR-based DNA fingerprinting methods.

**KEY WORDS** DNA fingerprinting, sharpshooter, *H. coagulata*

THE SHARPSHOOTER *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae) is a xylem-feeding leafhopper that is a serious pest because it vectors a strain of *Xylella fastidiosa*, a bacterium that causes Pierce's disease of grapevines (*Vitis vinifera* L. and *Vitis labrusca* L.) (Hopkins and Mollenhauer 1973). This sharpshooter is native to the southern United States, from Florida to Texas, and it is also distributed in Northern Mexico (Turner and Pollard 1959, Nielsen 1968, Brlansky et al. 1983). Within the last 10 yr, *H. coagulata* has established in southern California where it poses a potentially serious threat to the wine and table grape industry in that region (Sorensen and Gill 1996). Pierce's disease is caused when *X. fastidiosa* resides, multiplies, and interferes with the water conductive system or xylem of the plant, initially causing dieback of leaves and shoots, and eventually causing the entire plant to collapse and die within a year or two (Hopkins 1989, Varela et al. 2001). Strains of this bacterium, which are vectored by several species of sharpshooters, including *Homalodisca insolita* (Walker) and *Homalodisca liturata* (Ball) (Turner and Pollard 1959, Mizell and French 1987), have also been associated with other diseases, such as leaf scorches of plum (Kitajima et al. 1975); almond (Mircetich et al. 1976); maple (Sherald et al. 1987); oleander (Opgenorth 1995; Purcell et al. 1999; Costa el at. 2000); coffee (Lima et al. 1998); elm, sycamore, and oak (Hearon et al. 1980); periwinkle wilt (Brlansky et al. 1983); ragweed stunt and citrus variegated chlorosis (Roistacher 1992, Chang et al. 1993, Derrick and Timmer 2000); and phony peach disease (Hopkins et al. 1973). Pierce's disease, however, may be the most economically significant as it is the principal factor preventing the development of a grape industry in the southeastern United States based on the high-quality *V. vinifera* and *V. labrusca* grapes (Hopkins 1987).

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DNA markers have proved to be valuable tools for population genetic studies because they can assist in determining geographic variation, identity of origin, gene flow mechanisms, and effects of management practices. In addition, polymorphic markers can be used for investigating the organization of genomes and for the construction of genetic maps, which can provide strategies for gene isolation (Narang et al. 1993, Karp and Edwards 1997).

A sensitive approach for obtaining polymorphic DNA markers is based on the use of simple sequence repeats (SSR) or microsatellites. Microsatellites are ubiquitous in eukaryotic genomes and can be found in both protein-coding and -noncoding regions (Tóth et al. 2000). Microsatellites are widely used as genetic markers because they are codominant, multiallelic, easily scored, and highly polymorphic, although drawbacks for their use are the time and cost required to characterize them (Karp and Edwards 1997). However, three DNA fingerprinting procedures, inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) (Zietkiewicz et al. 1994) and primer pair-ISSR-PCR (pp-ISSR-PCR) (Prevost and Wilkinson 1999, Cekic et al. 2001), randomly amplified microsatellite polymorphisms (RAMP) (Wu et al. 1994), and selective amplification of microsatellite polymorphic loci (SAMPL) (Witsenboer et al. 1997), permit detection of DNA variation in microsatellites without the need to isolate and sequence specific DNA fragments. The approach for these techniques involves amplification with oligonucleotide primers corresponding directly to random SSR sites. This involves the use of 5′-anchored or compound ISSR primers where the anchor serves to fix the annealing of the primer to a single position of the target site, thus resulting in a low level of slippage during amplification. These primers generate products that incorporate the upstream boundary of the SSR and therefore generate coamplified products capable of carrying polymorphisms that may be present at the SSR target site. With the random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR) (Williams et al. 1990) and primer pair-RAPD-PCR (pp-RAPD-PCR) methods (Yasukochi 1998), amplification of genomic DNA by using short random decamer primers results in multiple amplification products from loci distributed throughout the genome.

The objective of the current study was to develop molecular genetic markers for *H. coagulata* by PCR-based DNA fingerprinting methods to detect DNA polymorphisms or genetic variation in natural populations. We applied multi-locus PCR-based DNA fingerprinting methods that included ISSR-PCR and pp-ISSR-PCR, RAMP, SAMPL, and pp-RAPD-PCR. Markers generated by these methods are scored as dominant. However, if family relationships are available or known, methods (ISSR-PCR and pp-ISSR-PCR, RAMP, and SAMPL) incorporating SSR are capable of identifying codominant markers if using 5′-anchored or compound ISSR primers (reviewed in Karp and Edwards 1997). Modifications in selected DNA fingerprinting methods included, for pp-ISSR-PCR, primer pair combinations of 5′-anchored ISSR primers and combinations of 5′-anchored and compound ISSR primers, and for RAMP, primer pair combinations of compound ISSR primers and RAPD primers. We developed DNA markers with PCR-based DNA fingerprinting methods and demonstrated the ability of these markers to detect geographic variation in populations of glassy-winged sharpshooters from Bakersfield and Riverside, CA and Weslaco, TX. In addition, we demonstrated the ability of the DNA fingerprinting methods to distinguish between three *Homalodisca* sharpshooter species.

## Materials and Methods

**Insect Collection.** Adult *H. coagulata* were collected on Texas mountain laurel, crepe myrtle, and grapevines in midsummer and early December 2001 in Weslaco, TX (Hidalgo County). Live insects were frozen overnight at −70°C and than transferred to 95% nondenatured ethanol and stored at −70°C. *H. coagulata* from Bakersfield, CA, (Kern County) were from an F0 culture at USDA–APHIS, Mission Plant Protection Center, Moore Air Field. *H. coagulata* and *H. liturata* from Riverside, CA (Riverside County), were collected at Agricultural Operations, University of California Riverside, from mixed citrus (*H. coagulata*) and irrigated native vegetation (*H. liturata*). *H. insolita* were collected in Quincy, FL (Leon County).

**Genomic DNA Isolation.** High-molecular-weight genomic DNA was extracted according to standard methods (Sambrook and Russell 2001). Individual whole sharpshooters were homogenized in 2 ml of lysis buffer with one 20-s burst (Tissue Tearor, Biospec Products, Inc., Bartleville, OK). The integrity of the DNA was determined by loading it onto 1% agarose gels and submitting it to electrophoresis in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA) in the presence of 0.2 μg/ml ethidium bromide. The concentration of the genomic DNA was estimated either spectrophotometrically ($A_{260}$) or with the DNA Mass Ladder (Invitrogen, Carlsbad, CA).

**ISSR-PCR.** ISSR-PCR reactions (Gupta et al. 1994, Zietkiewicz et al. 1994) were performed in a final volume of 20 μl with the following components with some modifications: 1× PCR buffer [50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 (p-6 and p-9 and pp-ISSR-PCR #10) or 1.8 mM $MgCl_2$, and 0.01% gelatin], 0.25 mM deoxynucleotide triphosphates, 0.25 μM ISSR primer(s) [3′-, 5′-anchored, or compound primers (only) that are reported from various sources (Wu et al. 1994, Zietkiewicz et al. 1994, Fisher et al. 1996, Witsenboer et al. 1997)] (Table 1), 0.5–1.0 ng of high-molecular-weight genomic DNA and 0.05 U/μl *Taq*DNA Polymerase (New England Biolabs, Beverly, MA). Primer KKVRVRV$(CT)_6$ (Fisher et al. 1996) was modified to include a *TG* dinucleotide repeat motif, KKVRVRV$(TG)_6$ and primer CCGG$(T)_7$ reported by Wu et al. (1994) was modified to extend the *T* mononucleotide repeat motif CCGG$(T)_{14}$. Notations: K =

**Table 1. Summary of successful reactions showing primers and primer pairs utilized in the DNA fingerprinting methods screening procedure**

| ISSR primer no. | Primer | Tm | No. polym markers | pp-ISSR-PCR reaction no. | ISSR primer pairs | Tm | No. polym markers |
|---|---|---|---|---|---|---|---|
| p-6 | $HVH(TG)_7T$ | 56° | 11 | 2 | $HVH(TG)_7T$ | 59° | 10 |
| p-9 | $CCAG(GT)_7$ | 52° | 8 | | $CCAG(GT)_7$ | | |
| p-10 | $G(TG)_4(AG)_4A$ | 41° | 4 | 4 | $KKVRVRV(TG)_6$ | 50° | 6 |
| p-13 | $A(CA)_7(TA)_2T$ | 54° | 7 | | $CCAG(GT)_7$ | | |
| p-15 | $T(GT)_7(AT)_2$ | 52° | 4 | 7 | $KKVRVRV(TG)_6$ $C(CT)_4(GT)_4G$ | 47° | 8 |
| | | | | 8 | $(TCG)_4GY$ $A(CA)_7(TA)_2T$ | 50° | 3 |
| | | | | 9 | $CCAG(GT)_7$ $T(GT)_7(AT)_2$ | 50° | 9 |
| | | | | 10 | $HVH(TG)_7T$ $T(GT)_7(AT)_2$ | 57° | 5 |

| RAMP Reaction no. | Compound ISSR primer | RAPD primer | RAPD sequence | Tm | No. polym markers |
|---|---|---|---|---|---|
| 3 | $C(CT)_4(GT)_4G$ | OPA-18 | AGGTGACCGT | 50° | 9 |
| 13 | $A(CA)_7(TA)_2$ | OPA-03 | AGTCAGCCAC | 50° | 6 |
| 15 | $A(CA)_7(TA)_2$ | OPA-18 | | 45° | 5 |
| 16 | $C(AC)_4(AG)_4A$ | OPA-03 | | 43° | 4 |
| 17 | $C(AC)_4(AG)_4A$ | OPA-10 | GTGATCGCAG | 41° | 5 |
| 18 | $C(AC)_4(AG)_4A$ | OPA-18 | | 41° | 4 |
| 48 | $A(CA)_7(TA)_2$ | OPM-02 | ACAACGCCTC | 42° | 4 |
| 54 | $G(TG)_4(AG)_4A$ | OPM-02 | | 43° | 7 |
| 71 | $C(AC)_4(AG)_4A$ | OPJ-06 | TCGTTCCGCA | 41° | 3 |
| 72 | $C(AC)_4(AG)_4A$ | OPM-02 | | 43° | 4 |
| 75 | $C(AC)_4(AG)_4A$ | OPV-14 | AGATCCCGCC | 41° | 5 |
| 78 | $T(GT)7(AT)_2$ | OPM-02 | | 42° | 2 |

| SAMPL reaction no. | Adapter primer | ISSR primer | Tm | No. poym markers | pp-RAPD-PCR reaction no. | RAPD primers | No. polym markers |
|---|---|---|---|---|---|---|---|
| 7 | M + CTC | $KKVRVRV(TG)_6$ | 53° | 3 | 1 | OPA-03/A-10 | 4 |
| 8 | M + CTC | $G(TG)_4(AG)_4A$ | 49° | 2 | 2 | OPA-03/A-18 | 2 |
| 12 | M + CAT | $G(TG)_4(AG)_4A$ | 49° | 1 | 6 | OPA-03/M-02 | 1 |
| 15 | M + CTC | $A(CA)_7(TA)_2$ | 49° | 3 | 10 | OPA-10/A-18 | 3 |
| 16 | M + CTC | $C(AC)_4(AG)_4A$ | 43° | 2 | 13 | OPA-10/J-06 | 5 |
| 19 | M + CAT | $C(AC)_4(AG)_4A$ | 50° | 2 | 17 | OPA-10/V-14 | 7 |
| 23 | M + CTC | $CCGG(T)_{14}$ | 45° | 5 | 20 | OPA-18/J-06 | 6 |
| 25 | M + CTC | $CCAG(GT)_7$ | 50° | 3 | 21 | OPA-18/M-02 | 2 |
| 27 | E + ACA | $G(TG)_4(AG)_4A$ | 49° | 3 | 33 | OPJ-01/M-20 | 2 |
| 34 | E + AGC | $C(AC)_4(AG)_4A$ | 58° | 4 | 36 | OPJ-06/M-02 | 1 |
| 36 | E + AGC | $T(GT)_7(AT)_2$ | 64° | 4 | 39 | OPJ-06/V-14 | 4 |
| | | | | | 42 | OPM-02/V-14 | 3 |

Reactions were performed with genomic DNA from three individual *H. coagulata* per reaction (46 total) from Weslaco, TX. pp, methods incorporating primer pairs. RAPD primer sequences not in the table: OPJ-01 (CCCGGCAATA) and OPM-20 (AGGTCTTGGG). No. Polym, number of polymporphic markers. Notations: K, G/T; V, G/C/A; R, G/A; H, A/T/C; and V, G/C/A.

G/T, V = G/C/A, and R = G/A and for primer $HVH(TG)_7T$ (Zietkiewicz et al. 1994), H = A/T/C and V = G/C/A. The cycling parameters were as follows: one cycle at 94°C for 2 min followed by 45 cycles at 94°C for 1 min (denaturation), specified Tm for 1 min (annealing), and 72°C for 2 min (extension) (Table 1). Reactions were optimized for amount of genomic DNA, $MgCl_2$ concentration, and cycle number. Negative control reactions were performed in the absence of genomic DNA. Amplification products were loaded onto 2% TBE agarose gels and submitted to electrophoresis in 1× TBE buffer (90 mM Tris-borate, 2 mM EDTA) in the presence of 0.2 μg/ml ethidium bromide. Photographs of the gels were taken with the Chemi Doc System and markers/bands were scored with the Quantity One Software (Bio-Rad, Hercules, CA).

**RAMP.** RAMP reactions (Wu et al. 1994) were modified to include compound ISSR primers instead of 5′-anchored primers. The amplification reactions were performed as with ISSR-PCR except that the $MgCl_2$ concentration was 2.0 mM and a compound ISSR primer [only the compound primers reported in Witsenboer et al. (1997)] was combined with a decamer RAPD primer (Operon Technologies, Inc., Alameda, CA), with each primer combination having its specific annealing temperature determined experimentally (Table 1). Specific annealing temperatures

promoted recovery of a greater number of markers than asymmetric PCR cycling (Wu et al. 1994, Matsumoto et al. 1998). Amplification products were processed as for ISSR-PCR described above, with 2% TBE agarose gels in the presence of ethidium bromide.

**SAMPL.** High-molecular-weight genomic DNA (0.5 $\mu$g) was digested with 5 U each of *Eco*RI and *Mse*I endonuclease enzymes (Invitrogen, Carlsbad, CA) for 2 h at 37°C in REact buffer 1 [50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$] in a final volume of 40 $\mu$l with some modifications (Vos et al. 1995, Witsenboer et al. 1997). Before ligation, double-stranded *Eco*RI and *Mse*I adapters were prepared to final concentrations of 5 and 50 pmol/$\mu$l, respectively, by combining the appropriate complementary single-stranded oligonucleotides, heating them at 95°C for 7 min, and allowing them to cool to room temperature (2 h). The ligation mixture consisted of the *Eco*RI/*Mse*I digest, 5 and 50 pmol of *Eco*RI and *Mse*I adapters, respectively, 1× DNA ligase reaction buffer [50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 5 mM dithiothreitol, 5% (wt:vol) polyethylene glycol-8000], and 1 U of $T_4$ DNA ligase (Invitrogen) in a final volume of 60 $\mu$l. The ligation reaction was incubated at 37°C for 3 h and diluted 4.17-fold with TE [10 mM Tris-HCl (pH 7.5), 1 mM EDTA]. This mixture served as template for first-round amplification in which 12.5 $\mu$l (or 25 ng of ligated DNA) was used with the components described for ISSR-PCR with primers E + 0 (AGACTGCGTACCATTTC) and M + 0 (GATGAGTCCTGAGTAA) each at 0.25 $\mu$M final concentration with 2.0 mM $MgCl_2$. The following cycling parameters were used: one cycle at 94°C for 2 min followed by 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min. The first-round amplification template was diluted 10-fold with TE and stored at −20°C and served as template for second-round amplification. Second-round amplification was performed as for the ISSR-PCR reactions with 1.5 mM $MgCl_2$, but one primer was targeted at either the *Eco*RI or *Mse*I adapter sequences with three nucleotide extensions, and the other was a 5′-anchored or compound ISSR primer (Table 1). All primers were at a final concentration of 0.25 $\mu$M. The cycling parameters were as for ISSR-PCR with a specific annealing temperature per primer set (Table 1), which generated a greater number of markers than touch-down cycling (Witsenboer et al. 1997). Amplification products were processed as for ISSR-PCR above, with 2% TBE agarose gels in the presence of ethidium bromide.

**RAPD-PCR.** RAPD-PCR reactions (Williams et al. 1990) incorporating primer pairs, pp-RAPD-PCR (Yasukochi 1998) were performed as for ISSR-PCR with 2 mM $MgCl_2$ and 0.25 pmol/$\mu$l decamer primers (Table 1) at a Tm of 36°C.

**Amplification of the 16S rDNA gene of *X. fastidiosa*.** Gene-specific amplification reactions were performed with the standard primer sets of Minsavage et al. (1994) (RST31, GCGTTAATTTTCGAAGTGATTCGA; RST33, CACCATTCGTATCCCGGTG; Tm 55°C) and Firrao and Bazzi (1994) (XF1, CAGCACATTGGTAGTAATAC; XF6, ACTAGGTATTAACCAATTGC; Tm 50°C). The reaction components were as with ISSR-PCR with template DNA ranging from 2.5 to 16 ng and 40 *H. coagulata* from different natural populations. The reactions were allowed to proceed for 40 cycles. Two-rounds of amplification were attempted in one insect by using 1, 3, and 5 $\mu$l of the first-round template. Each round was amplified for 30 cycles. Positive control reactions were performed with two strains of *X. fastidiosa* (ATCC 700964 and 35881; Wells et al. 1987) genomic DNA (donated by Ruiz and Vacek, USDA–APHIS, Mission Plant Protection Center, Moore Air Field; purchased from American Type Culture Collection, Manassas, VA). Amplification reactions were performed with the abovementioned *X. fastidiosa* primer sets with 5.0 ng each of genomic DNA. The reactions were amplified for 40 cycles.

**Data Analysis.** Bands observed in each lane were compared with all the other lanes of the same gel and only the most distinguishable and reproducible bands were scored as presence (1) or absence (0). Fragment sizes were estimated based on the 1.0 kb Plus DNA Ladder (Invitrogen) according to the algorithm provided in the Quantity One software. Genetic variation was analyzed with the POPGENE 3.2 genetic software program (Yen et al. 1996). Markers generated were treated as dominant markers. The program estimates polymorphic loci, percentage of polymorphic loci, and gene diversity (Nei 1973, 1977). Dendrograms based on Nei's (1978) genetic distance and unweighted pair-group method with arithmetic average of Sneath and Sokal (1973) were constructed with the program Tools for Population Genetic Analyses (TPFGA, version 1.3; Miller 1997). Bootstrapping over loci was also performed with TPFGA with 1000 permutations by the algorithm of Felsenstein (1985).

## Results

**DNA Fingerprinting Screening Procedure.** To develop molecular genetic markers for *H. coagulata* to detect DNA polymorphisms or genetic variation, we initiated a small-scale DNA fingerprinting screening procedure with three insects. Initially, one insect was used to determine which primers or primer pairs (210 total) amplified in a template-specific manner with *H. coagulata* genomic DNA with the four methods. Next, three insects (insect set) per primer or primer pair (46 total) were used to estimate the sensitivity and efficiency of each method. Reactions that were not consistent after several independent amplifications were eliminated. The successful DNA fingerprinting reactions per insect set per method, including the description of the primers or primer pair combinations, the annealing temperatures (Tm), and the number of markers generated per reaction are shown on Table 1. Individual ISSR-PCR primer reactions for pp-ISSR-PCR and individual ISSR-PCR and RAPD-PCR primer reactions for RAMP were performed to confirm that the banding patterns were generated by the incorpo-

**Table 2. Summary of the DNA fingerprinting methods screening procedure**

| Method | No. primers screened | No. primers amplified | No. polym. markers | Efficiency ratio | Screening efficiency |
|---|---|---|---|---|---|
| ISSR-PCR | 15 | 5 | 34 | 6.80 | 2.27 |
| pp-ISSR-PCR | 17 | 6 | 41 | 6.83 | 2.41 |
| RAMP | 93 | 12 | 58 | 4.83 | 0.62 |
| SAMPL | 40 | 11 | 32 | 2.91 | 0.80 |
| pp-RAPD-PCR | 45 | 12 | 40 | 3.33 | 0.88 |
| Total | 210 | 46 | 205 | | |

ration of both primers into each reaction. This step eliminated all of the 5′-anchored ISSR primers tested with RAMP. Table 2 summarizes the results of the screening procedure. In total, 205 polymorphic markers were generated with the four methods with ISSR-PCR and pp-ISSR-PCR, RAMP, SAMPL, and pp-RAPD-PCR producing 34 and 41, 58, 32, and 40 polymorphic markers, respectively. The efficiency ratio (number of polymorphic markers per number of primers amplified) of each method was as follows: 6.83 (pp-ISSR-PCR) and 6.80 (ISSR-PCR), 4.83 (RAMP), 3.33 (pp-RAPD-PCR), and 2.91 (SAMPL). The highest screening efficiency ratio values (number of polymorphic markers per number of primers or primer pairs screened) were seen with pp-ISSR-PCR (2.41) and ISSR-PCR (2.27).

**Detection of DNA Polymorphisms in a Natural Population.** To determine the ability of selected reactions of the DNA fingerprinting methods in detecting DNA polymorphisms or genetic variation, 10–30 *H. coagulata* from a natural population in Weslaco were used (Table 3). Depending on the sample size, the number of polymorphic loci ranged from five (pp-RAPD-PCR #6) to 32 (ISSR-PCR p-13), and the percentage of polymorphic loci was 100% for most primers or primer pairs per method. Gene diversity ranged from 0.10 to 0.26 for ISSR-PCR p-10 and pp-RAPD-PCR #6, respectively. Due to its complexity and low efficiency, SAMPL was not further pursued.

**Detection of Geographic Variation.** To determine the ability of selected reactions from the DNA fingerprinting methods to detect geographic variation in *H. coagulata*, 10 insects each from Bakersfield, Riverside, and Weslaco were used. Weslaco is ≈1,571 miles from Riverside and Bakersfield is ≈170 miles northwest of Riverside. Figure 1 visually demonstrates geographic variation in the three natural populations of *H. coagulata* with RAMP #54. Most bands were seen within the 300–500-bp region. Visual inspection shows that *H. coagulata* from Weslaco are associated with a more complex banding pattern. A dendrogram based on Nei's genetic distance of this reaction is illustrated in Fig. 2A. *H. coagulata* from Bakersfield and Riverside formed a cluster separate from Weslaco. Two other ISSR-PCR reactions tested with ISSR compound primers, p-10 (Fig. 2B) and p-13 (Fig. 2C) demonstrated geographic variation and in both cases, Bakersfield and Riverside also formed a cluster separate from Weslaco. Geographic variation of *H. coagulata* was also demonstrated with pp-RAPD-PCR #1 (Fig. 2D) and #17 (Fig. 2E). Dendrograms based on these reactions clustered Bakersfield and Weslaco with pp-RAPD-PCR #1 and Riverside and Weslaco with pp-RAPD-PCR #17. Estimation of the distribution of the geographic variation in the three *H. coagulata* populations with the previous reactions is demonstrated in Table 4. Single-populations analyses demonstrated the greatest genetic variation in the *H. coagulata* population from Weslaco with RAMP #54, ISSR-PCR p-10, and pp-RAPD-PCR #1. In contrast, higher genetic variation was detected in Bakersfield with pp-RAPD-PCR #17. Percentage of polymorphic loci in the multipopulations analyses ranged from 92.9 to 100.0% in all reactions. More geographic-specific markers (GSM)

**Table 3. Results from selected reactions from DNA fingerprinting methods showing genetic variation statistics from a natural population from Weslaco**

| Method | Primer (p) or reaction no. (#) | Sample size | Loci | P | %P | G. D. |
|---|---|---|---|---|---|---|
| ISSR-PCR | p-9 | 30 | 28 | 28 | 100.0 | 0.15 (0.12) |
| ISSR-PCR | p-10 | 30 | 25 | 25 | 100.0 | 0.10 (0.10) |
| ISSR-PCR | p-13 | 30 | 32 | 32 | 100.0 | 0.12 (0.09) |
| pp-ISSR-PCR | #7 | 10 | 15 | 14 | 93.3 | 0.17 (0.12) |
| RAMP | #54 | 10 | 15 | 15 | 100.0 | 0.23 (0.12) |
| RAMP | #75 | 30 | 21 | 21 | 100.0 | 0.20 (0.15) |
| SAMPL | #34 | 30 | 14 | 14 | 100.0 | 0.10 (0.07) |
| pp-RAPD-PCR | #1 | 10 | 11 | 10 | 90.9 | 0.19 (0.17) |
| pp-RAPD-PCR | #6 | 30 | 5 | 5 | 100.0 | 0.26 (0.16) |
| pp-RAPD-PCR | #17 | 30 | 15 | 15 | 100.0 | 0.17 (0.16) |

Refer to Table 1 for the description of primers and reaction numbers. P, polymorphic loci; %P, percentage polymorphic loci; and G. D., gene diversity (stand. dev.).

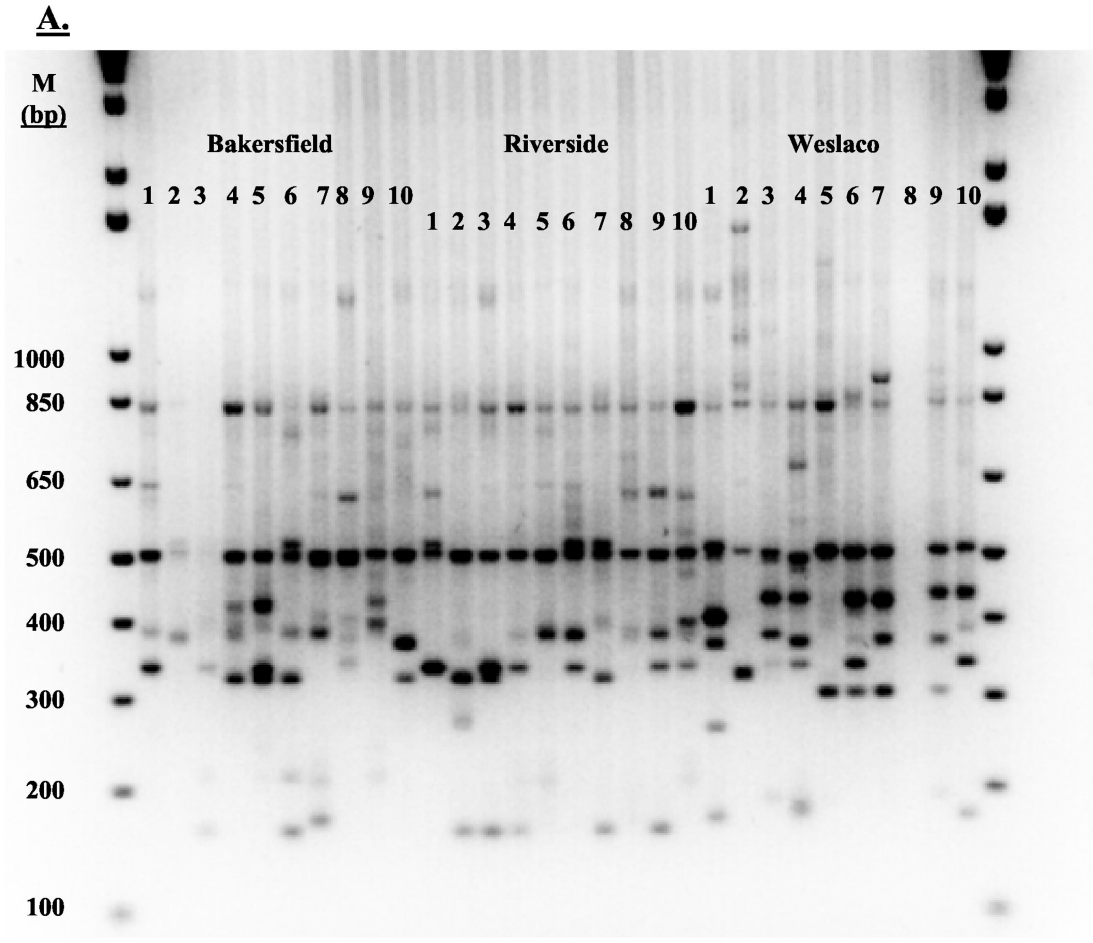


**Fig. 1.** Geographic variation in *H. coagulata.* RAMP #54 [ISSR p-10, $G(TG)_4(AG)_4A$, and RAPD primer OPM-02] was performed with genomic DNA from 10 insects each from Bakersfield, Riverside, and Weslaco. M, 1.0 Kb Plus DNA Ladder.

were detected in Weslaco with RAMP #54 (6), ISSR-PCR p-10 (10), and ISSR-PCR p-13 (9). Geographic-specific markers may be an indication of subdivided populations.

**Distinction of *Homalodisca* Sharpshooters.** To determine the ability of selected reactions of the DNA fingerprinting methods to detect DNA polymorphisms and to distinguish different species of *Homalodisca* sharpshooters, 10 individuals each of *H. coagulata*, *H. insolita*, and *H. liturata* were used. *H. coagulata* and *H. insolita* are both considered native to the southern United States, whereas *H. liturata* is distributed in Arizona and southern California (Young 1958, 1968). *H. coagulata* has only recently made its way into southern California (Sorensen and Gill 1996). Figure 3 demonstrates results from pp-ISSR-PCR #7. Species-specific bands are indicated by the boxed areas, which are labeled with their molecular weights (base pairs). Two species-specific markers were detected in *H. coagulata* at 1,419 bp (faintly) and 346 bp. Three species-specific markers (334, 252, and 163 bp) were detected in *H. liturata* and one in *H. insolita* (221 bp). Relationships among the three species for this reaction are illustrated graphically on a dendrogram (Fig. 4A). *H. coagulata* and *H. insolita* formed a cluster separate from *H. liturata.* Both of these species were also clustered together in two other ISSR-PCR reactions tested, p-10 (Fig. 4B) and p-13 (Fig. 4C). Dendrograms based on pp-RAPD-PCR DNA fingerprinting showed *H. coagulata* and *H. insolita* clustered with reaction #1 (Fig. 4D) in one case and *H. liturata* and *H. insolita* in another case with reaction #17 (Fig. 4E). Species-specific bands that could distinguish the three *Homalodisca* species were detected with these reactions. Estimation and distribution of genetic variation among the species is shown on Table 5. Single-populations analyses estimated higher genetic variation in *H. coagulata* with methods incorporating SSR, pp-ISSR-PCR #7, and ISSR-PCR p-10 and p-13. In contrast, higher variation was estimated in *H. liturata* with pp-RAPD-PCR #17. Percentage of polymorphic loci

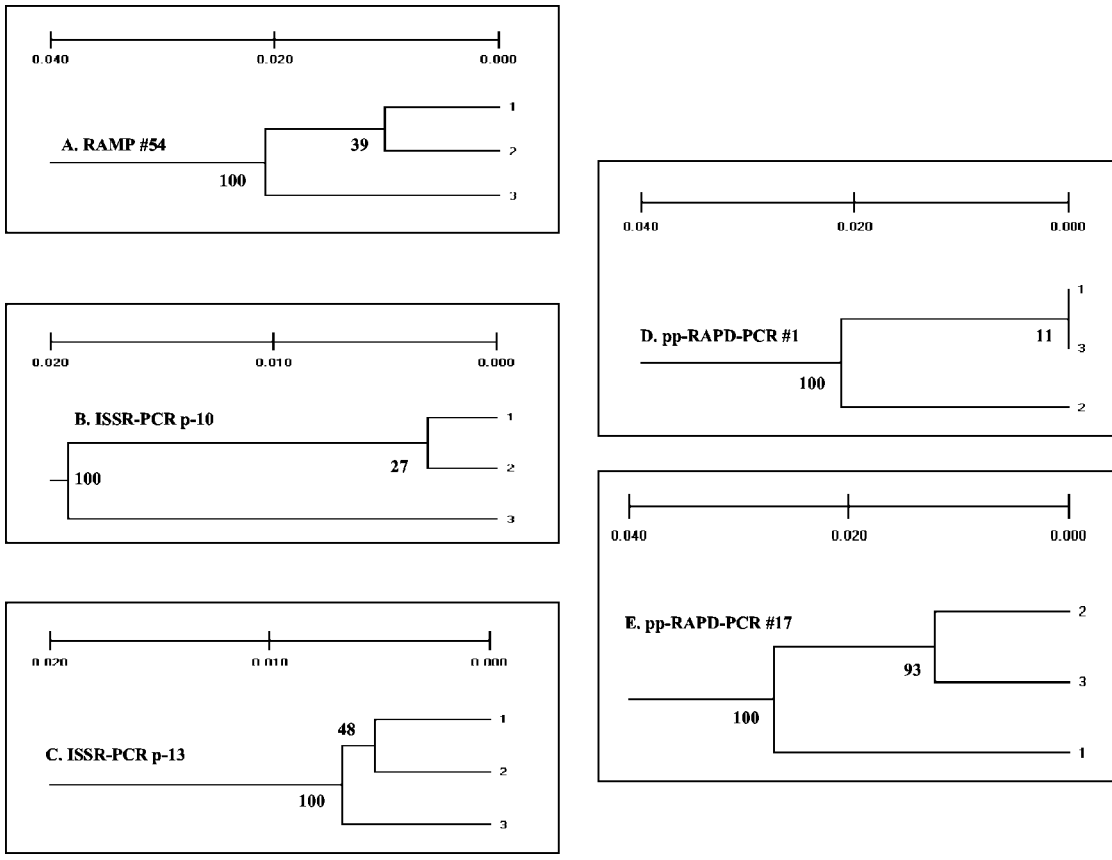


**Fig. 2.** Dendrograms based on Nei's genetic distance by the method of unweighted pair-group method with arithmetic average showing relationships of three *H. coagulata* geographic populations. Genomic DNA from 10 insects each from Bakersfield (1), Riverside (2), and Weslaco (3) was used for DNA fingerprinting with (A) RAMP #54 [ISSR p-10, $G(TG)_4(AG)_4A$, and RAPD primer OPM-02]; (B) ISSR-PCR p-10; and (C) ISSR-PCR p-13 [$A(CA)_7(TA)_2T$]. Dendrograms of geographic populations of *H. coagulata* based on pp-RAPD-PCR DNA fingerprinting were performed with (D) pp-RAPD-PCR #1 (OPA-03/A-10) and (E) #17 (OPA-10/V-14). Distances are indicated above the dendrograms and bootstrap support values are indicated at the nodes.

in the multipopulation analyses was 100% for all reactions.

**Amplification of the 16S rDNA Gene of *X. fastidiosa*.** It was possible that some of the banding patterns from ISSR-PCR or the other DNA fingerprinting methods were generated by the presence of *X. fastidiosa* in *Homalodisca* sharpshooters because whole insects were homogenized. Based on the principles of PCR, amplification with a random primer should favor the template that is present in higher amounts due to competition alone. In this case it was expected that the DNA template of the insect was present in much higher amounts than the template of the bacterium (if present). To confirm that the banding patterns seen with our DNA fingerprinting reactions were of insect origin, we performed amplification reactions with two separate standard *X. fastidiosa* primers sets (Firrao and Bazzi 1994, Minsavage et al. 1994) with template DNA ranging from 2.5- to 32-fold higher than normal use and 40 insects (*H. coagulata*). The amplification reactions using both sets of *X. fastidiosa*-specific primers, including the two-rounds of amplification, failed to show any amplification in all insects tested. However, amplification reactions with both strains of *X. fastidiosa* genomic DNA (ATCC 700964 and 35881), and both sets of primers produced positive products of the expected sizes (RST31/RST33, 733 bp; and XF1/XF6, 400 bp) for each bacterial strain (data not shown). With our present method, *X. fastidiosa* was not detected in genomic DNA isolated from whole insects; therefore, these results indicate that the banding patterns seen with the various DNA fingerprinting assays were generated from the insects and not the bacterium.

## Discussion

To our knowledge, the present work using various DNA fingerprinting procedures is the first to identify polymorphic molecular genetic markers (Table 2) and

**Table 4. Estimation of *H. coagulata* geographic single- and multipopulations (M-P) genetic variation statistics performed with 10 insects each from Bakersfield, Riverside, and Weslaco**

| Method | Location | P | %P | G. D. | GSM |
|---|---|---|---|---|---|
| RAMP #54 | Bakersfield | 10 | 55.6 | 0.16 (0.19) | 0 |
| | Riverside | 10 | 44.4 | 0.11 (0.15) | 1 |
| | Weslaco | 15 | 83.3 | 0.20 (0.14) | 6 |
| | M-P | 18 | 100.0 | 0.17 (0.12) | |
| ISSR-PCR p-10 | Bakersfield | 8 | 36.4 | 0.07 (0.12) | 2 |
| | Riversie | 7 | 31.8 | 0.09 (0.16) | 0 |
| | Weslaco | 16 | 72.7 | 0.14 (0.14) | 10 |
| | M-P | 21 | 95.5 | 0.11 (0.12) | |
| ISSR-PCR p-13 | Bakersfield | 16 | 59.3 | 0.09 (0.10) | 7 |
| | Riverside | 7 | 26.0 | 0.05 (0.11) | 1 |
| | Weslaco | 16 | 59.3 | 0.08 (0.08) | 9 |
| | M-P | 27 | 100.0 | 0.08 (0.08) | |
| pp-RAPD-PCR #1 | Bakersfield | 11 | 61.1 | 0.15 (0.07) | 4 |
| | Riverside | 8 | 44.4 | 0.08 (0.12) | 1 |
| | Weslaco | 12 | 66.7 | 0.14 (0.15) | 4 |
| | M-P | 18 | 100.0 | 0.14 (0.14) | |
| pp-RAPD-PCR #17 | Bakersfield | 10 | 71.4 | 0.13 (0.11) | 5 |
| | Riverside | 8 | 57.1 | 0.10 (0.13) | 2 |
| | Weslaco | 6 | 42.9 | 0.12 (0.19) | 1 |
| | M-P | 13 | 92.9 | 0.13 (0.15) | |

GSM, geographic-specific markers.

detect DNA polymorphisms in *H. coagulata* (Table 3), including two other *Homalodisca* sharpshooter species, *H. insolita* and *H. liturata* (Table 5). We chose DNA fingerprinting procedures that required no prior DNA sequence information because molecular genetic studies on this insect have not been reported or published to date. We established and confirmed the utility of a small-scale DNA fingerprinting screening procedure with four methods, ISSR-PCR and pp-ISSR-PCR, RAMP, SAMPL, and pp-RAPD-PCR. This is demonstrated by the detection of DNA polymorphisms and geographic variation in *H. coagulata* and by the distinction of *Homalodisca* sharpshooters. The advantage of this screening procedure is that it does not require many specimens, and it allows for rapid screening of several primers and several methods in a short amount of time. Generation of polymorphic markers with this approach gives a rapid estimation of the sensitivity and efficiency of each method for a specified DNA template of interest; in addition, more choices are generated to detect polymorphisms. Once potentially useful markers are identified by this approach, the markers can be further tested on larger natural populations to confirm the ability of these markers in detecting DNA polymorphisms.

Methods incorporating SSR seemed to be sensitive at detecting DNA polymorphisms in natural populations (Table 3); they were able to differentiate geographic populations of *H. coagulata* from California and Texas. *H. coagulata* from Bakersfield and Riverside formed a cluster that was separate from Weslaco in three independent DNA fingerprinting reactions (Fig. 2A–C), whereas pp-RAPD-PCR clustered them differently in each reaction (Fig. 2D and E). In addition, three reactions incorporating SSR clustered *H. coagulata* and *H. insolita* separately from *H. liturata* (Fig. 4A–C), but pp-RAPD-PCR clustered these two species differently in each reaction (Fig. 4D and E). The fact that methods incorporating SSR produced the above-mentioned clustering may be a coincidence because not all reactions from the DNA fingerprinting methods have been tested, so more testing is required before any conclusions can be drawn.

To our knowledge, this is the first time that RAMP analysis has been reported with compound ISSR primers, which were developed for SAMPL analysis by Witsenboer et al. 1997. Three of the five compound ISSR primers contained *GT* repeat motifs in their sequence, $G(TG)_4(AG)_4A$, $C(CT)_4(GT)_4G$, and $T(GT)_7(AT)_2$, and the complementary *CA* repeat motif was present in the other two compound ISSR primers, $A(CA)_7(TA)_2T$ and $C(AC)_4(AG)_4A$. Based on a survey of microsatellites in different genomes by Tóth et al. (2000), the most frequent repeat motif in Arthropoda (mainly *Drosophila*) in all regions of the genome, which include intergenic regions, introns, and exons is the dinucleotide repeat *AC*. This dinucleotide repeat motif is found in greater frequency in intergenic regions and introns than in exons. The second most frequent repeat motif for arthropods is the mononucleotide *A*. All of the ISSR primers (except the 3′-anchored ISSR primer) used for this study contained either a dinucleotide repeat unit consisting of *AC* or *GT* or a mononucleotide repeat unit consisting of *A* or *T* (Table 1). Amplification with a 3′-anchored ISSR primer containing a *CA* repeat motif, $(CA)_8RG$ (Zietkiewicz et al. 1994), was used successfully in ISSR-PCR, RAMP, and SAMPL reactions with *H. coagulata* genomic DNA (data not shown). RAMP has been used successfully to define genetic diversity in *Arabidopsis* (Wu et al. 1994), to construct a rat genetic map (Matsumoto et al. 1998), and to study the genetic diversity of barley (Sánchez de la Hoz et al. 1996, Dávila et al. 1999).

ISSR-PCR primer combinations produced consistent and reliable results. In combination with other

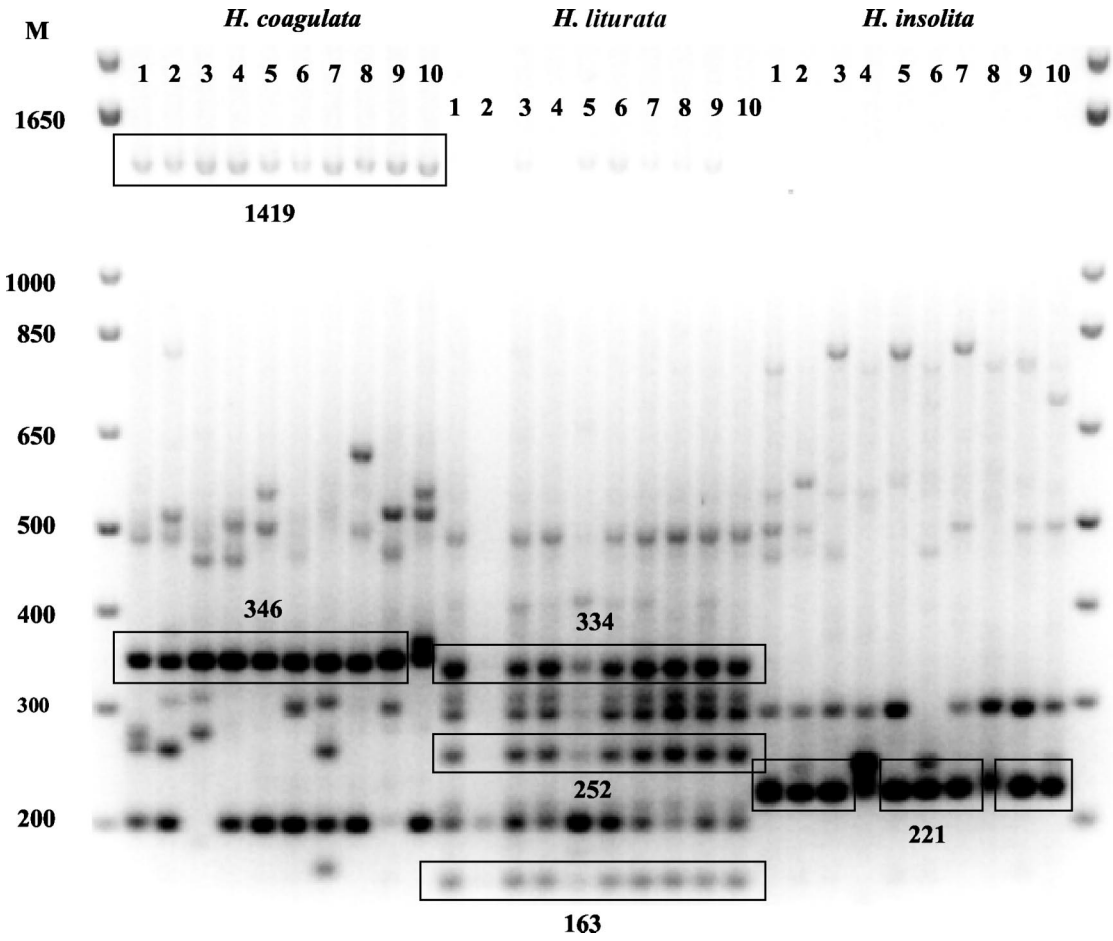


**Fig. 3.** Distinction of *Homalodisca* sharpshooters. Three *Homalodisca* species (10 each) *H. coagulata*, *H. liturata*, and *H. insolita* were used in pp-ISSR-PCR DNA fingerprinting with reaction #7 [ISSR primers KKVRVRV$(TG)_6$ and CCAG$(GT)_7$]. The boxed bands are species specific and are each labeled with their molecular weights (base pairs). M, 1.0 Kb Plus DNA Ladder.

ISSR primers, 5′-anchored ISSR primers HVH$(TG)_7$T and CCAG$(GT)_7$ and compound ISSR primers T$(GT)_4(AT)_2$ and C$(CT)_4(GT)_4$G were the most successful with *H. coagulata* genomic DNA. ISSR-PCR required the least amount of screening compared with the other PCR-based DNA fingerprinting methods (Table 2). ISSR-PCR has been used successfully for genetic characterization of the silkworm, *Bombyx mori* (L.) (Reddy et al. 1999), for genetic variability studies of *Biomphalaria straminea* snail complexes (Caldeira et al. 2001), and in a host of plant studies (reviewed by Wolfe and Liston 1998). pp-ISSR-PCR has been used successfully to fingerprint potato cultivars (Prevost and Wilkinson 1999) and for genetic linkage analysis in the seasonal flowering locus in *Fragaria* (Cekic et al. 2001).

The most successful compound ISSR primers in SAMPL analysis were C$(AC)_4(AG)_4$A and G$(TG)_4(AG)_4$A, which together produced 16 polymorphic SAMPL markers. However, the most successful 5′-anchored ISSR primer in SAMPL was CCGG$(T)_{14}$, which produced five polymorphic SAMPL markers. The mononucleotide (*T* or *A*) or the dinucleotide repeat motif (*GT* or *AC*) described above was also present in these primers. SAMPL has been successful in defining allelic diversity in lettuce (Witsenboer et al. 1997) and in assessing genetic variation among cowpea, *Vigna unguiculata* (L.) Walp. (Tosti and Negri 2002).

In our RAPD-PCR studies, amplification with a primer pair or combination provided more reliable results than with a single primer. For this reason, we chose not to pursue RAPD-PCR by using a single primer and decided to develop RAPD-PCR markers incorporating a pair of decamer primers (pp-RAPD-PCR). The RAPD-PCR procedure has been extremely useful for a variety of applications, including detecting DNA polymorphisms and constructing molecular genetic maps (Williams et al. 1990, Black et al. 1992), demonstrating phylogenetic relationships (Puterka et al. 1993), resolving genetic variability (Haymer and

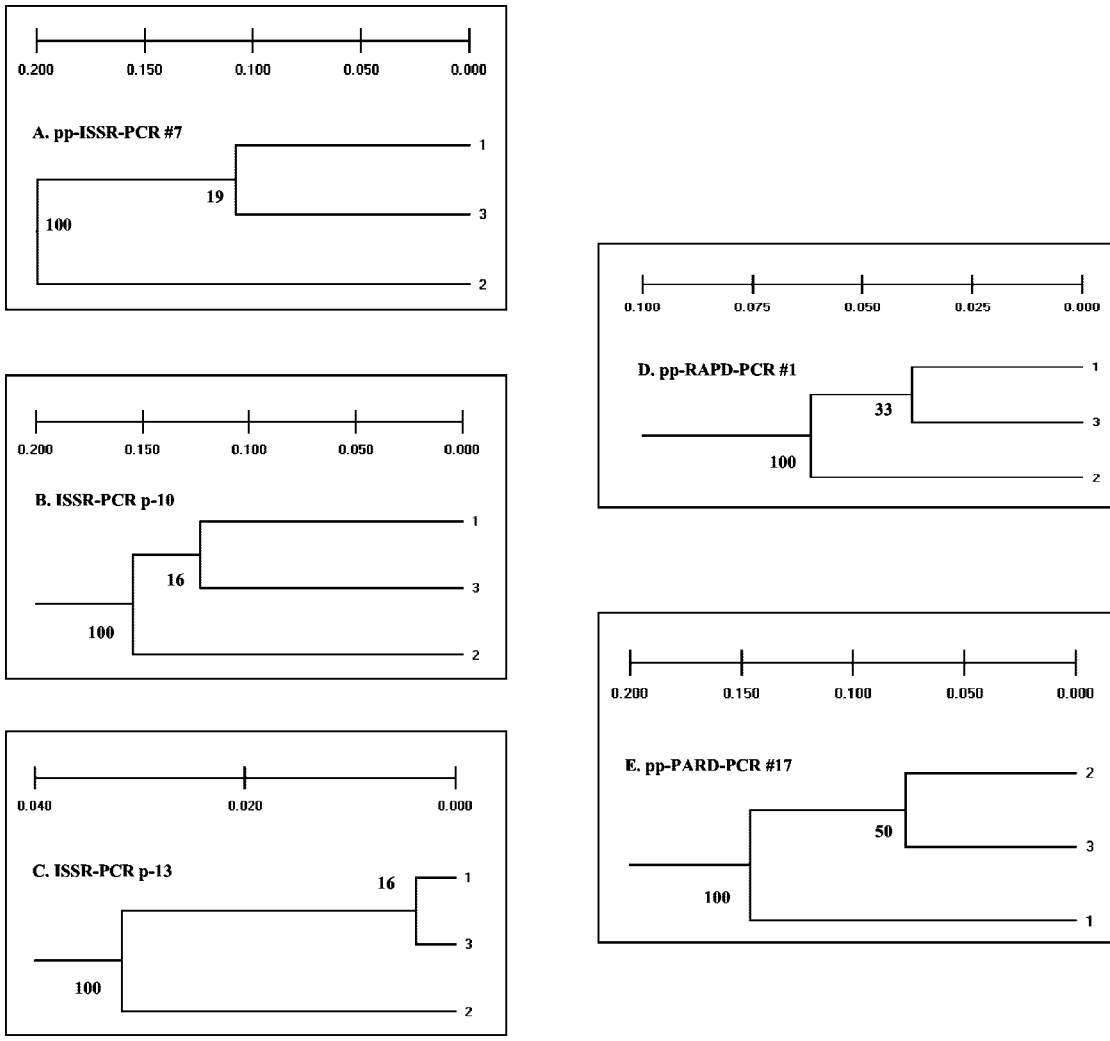


**Fig. 4.** Dendrograms based on Nei's genetic distance showing relationships of three *Homalodisca* species [*H. coagulata* (1), *H. liturata* (2), and *H. insolita* (3)]. Ten insects per species were used in (A) pp-ISSR-PCR #7 [ISSR primers KKVRVRV$(TG)_6$ and CCAG$(TG)_6$]; (B) ISSR-PCR p-10 [G$(TG)_4(AG)_4$A]; and (C) ISSR-PCR p-13 [A$(CA)_7(TA)_2$T]. Dendrograms of the three *Homalodisca* sharpshooters based on pp-RAPD-PCR DNA fingerprinting were performed with (D) pp-RAPD-PCR #1 (OPA-3/A-10) and (E) #17 (OPA-10/V-14). Distances are indicated above the dendrograms and bootstrap support values are indicated at the nodes.

McInnis 1994), and assessing genetic diversity (Nagararju and Singh 1997). The pp-RAPD-PCR procedure has been used to construct a genetic map of the silkworm (Yasukochi 1998) and a genetic linkage map of Einkorn wheat (Kojima et al. 1998).

In conclusion, DNA fingerprinting reactions (46) were identified that amplified in a template-specific manner with *H. coagulata* genomic DNA. The small-scale DNA fingerprinting methods screening procedure estimated ISSR-PCR and pp-ISSR-PCR to be the most efficient methods. Even though the SAMPL procedure incorporates SSR-targeted primers, it initially requires a higher amount of genomic DNA for digestion with the two restriction enzymes, although downstream steps require little template to complete the procedure. This characteristic may limit the use of SAMPL analysis when working with extremely small insects. The ISSR-PCR and pp-ISSR-PCR, RAMP, and RAPD procedures are straightforward because no manipulation of the genomic DNA is required. However, the use of SSR or microsatellites should make ISSR-PCR and pp-ISSR-PCR and RAMP more sensitive procedures. The present work sets the stage for performing large-scale population genetic studies of *H. coagulata*, such as estimating genetic variation within and among populations, identifying the origin of individuals, estimating gene flow and genetic structure, as well as construction of molecular genetic maps. A

**Table 5. Estimation of single- and multipopulations (M-P) genetic variation statistics performed with 10 insects each of *H. coagulata*, *H. insolita*, and *H. liturata***

| Method | Species | P | %P | G. D. |
|---|---|---|---|---|
| pp-ISSR-PCR #7 | *H. coagulata* | 17 | 65.4 | 0.13 (0.13) |
| | *H. insolita* | 4 | 15.4 | 0.07 (0.17) |
| | *H. liturata* | 5 | 19.2 | 0.06 (0.17) |
| | M-P | 26 | 100.0 | 0.19 (0.17) |
| ISSR-PCR p-10 | *H. coagulata* | 11 | 59.7 | 0.10 (0.13) |
| | *H. insolita* | 3 | 15.8 | 0.05 (0.11) |
| | *H. liturata* | 4 | 21.0 | 0.07 (0.16) |
| | M-P | 19 | 100.0 | 0.16 (0.15) |
| ISSR-PCR p-13 | *H. coagulata* | 17 | 53.1 | 0.08 (0.08) |
| | *H. insolita* | 8 | 25.0 | 0.05 (0.11) |
| | *H. liturata* | 12 | 37.5 | 0.06 (0.10) |
| | M-P | 32 | 100.0 | 0.08 (0.10) |
| pp-RAPD-PCR #1 | *H. coagulata* | 12 | 36.4 | 0.07 (0.12) |
| | *H. insolita* | 7 | 21.1 | 0.07 (0.15) |
| | *H. liturata* | 16 | 48.5 | 0.14 (0.19) |
| | M-P | 33 | 100.0 | 0.13 (0.10) |
| pp-RAPD-PCR #17 | *H. coagulata* | 5 | 50.0 | 0.13 (0.17) |
| | *H. insolita* | 3 | 30.0 | 0.14 (0.23) |
| | *H. liturata* | 5 | 50.0 | 0.18 (0.20) |
| | M-P | 10 | 100.0 | 0.22 (0.15) |

large-scale population genetic analysis study with populations of *H. coagulata* from several regions of the United States is in progress.

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